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Biodecolorization of textile dyes by immobilized enzymes in a vertical bioreactor system

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Abstract

A capacity of immobilized enzymes from *Trametes versicolor* U97 or *Pestalotiopsis* sp. NG007 to decolorize three textile dyes was investigated in a vertical bioreactor system. Immobilization was conducted using a double layer of alginate bead (1.5% w/w) and crude enzymes. The effect of mediators: Tween 80, 1-hydroxybenzotriazol (HBT), or 2,2,6,6-tetramethylpiperidin-1-yl)oxyl (TEMPO) was investigated on decolorization rate. The results showed that application of immobilized enzymes in a vertical bioreactor enhanced the decolorization efficiency of dyes in the order LB16>RRV9>RRN4, respectively. The decolorization rate was accelerated when a mediator TEMPO was used in immobilized enzyme of U97 or a Tween 80 was used in NG007. Reaction with glutaraldehyde (0.6%) for 4 h maintained the longevity and reusability of the beads. The study suggests that double layer immobilized enzyme in a bioreactor system has a terrific potential strategy for treating the textile dye effluents.

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Keywords: biodecolorization; bioreactor; immobilized enzymes; *Pestalotiopsis* sp; textile dyes; *Trametes versicolor*

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1. Introduction

Water pollution by waste dye stuff released from textile industries and dye houses represents a major environmental concern. It is estimated around 10-15% of textile dyes are lost during the dyeing process and 2-20% are directly discharged as aqueous effluents in different environmental components [1]. Unavoidably, these textile effluents would be a significant threat to public and environmental health[2]. The conventional technology based on physical or chemical treatments is usually effective only if the effluent volume is small [3]. In addition, a biological treatment in liquid state fermentation is less effective of removing dyes from continuous effluents [3]. Recently, enzymatic process has attracted much attention in the treatment of textile dyes in wastewater due to their eco-friendly and offers a rapid decolorization process [4, 5]. However, few studies have investigated the use of bioreactor system to decolorize these effluents. The purposes of this study are to investigate the potential use of a bioreactor system developed with double layer immobilized enzymes to decolorize textile dyes, to investigate the effect of enzymatic mediator addition in the second layer of the bead on decolorization rate of dyes, and finally, to investigate the longevity of the bead in sequential batch cycles decolorization in order to establish a bioreactor system that allows decolorization of reactive dyes over an extended period without addition of new enzymes.

2. Materials and method

2.1. Chemicals

Textile dyes Lefavix Blue 16 (LB16), Reactive Remazol Violet 9 (RRV9), and Reactive Remazol Navy 4 (RRN4) were used as substrates. *Trametes versicolor* U97 and *Pestalotiopsis* sp. NG007 were isolated from Ehime Prefecture, Japan. Tween 80, manganese (II) sulfate, hydrogen peroxide, 1-hydroxybenzotriazole (HBT), (2,2,6,6-tetramethylpiperidin-1-yl)oxyl (TEMPO), and glutaraldehyde were purchased from Wako Pure Chemical Industries, Ltd. (Japan).

2.2. Crude enzyme extraction

Isolate *Trametes versicolor* U97 or *Pestalotiopsis* sp. NG007 was pre-grown in wood meals (1 kg) containing glucose (100 g) and *shitake* nutrient (150 g) with final water content 60% for 1 month. Crude enzyme was extracted from the pre-grown fungus using homogenizer 10000 rpm for 10 minutes with malonate buffer pH 4.5. After filtration, filtrate was subjected to centrifuge at 8000 rpm, 4^{o}C for 20 minutes. Supernatant was collected and placed in container. Ammonium sulfate 75% was added into container, stirred for 1 hour, then centrifuged at 8000 rpm, 4^{o}C for 20 minutes. Pellet was collected and dilute in the buffer while stirred. The powder of crude enzyme was obtained after freeze-drying for 3 days.

2.3. Immobilization of crude enzymes

Immobilization of crude enzyme was conducted using double layer of alginate bead (1.5% *w/w*) and crude enzyme [0.36 U/mL] for *T. versicolor* or *Pestalotiopsis* sp. Mixture of Mn^{2+} (1 mM), H_2O_2 (1 mM) and Tween 80 (1%) was performed in the second layer. The effect of ligninolytic redox mediator such as HBT (1 mM) and/or TEMPO (1 mM) was investigated on decolorization rate. The chemical structure of Tween 80, HBT and TEMPO are shown in Fig 1.

2.4. Decolorization of textile dyes using bioreactor

Experiment was conducted using a bioreactor system MasterFlex®L/S® (Cole-Parmer Instrument Company) with two easy-loaders (model 7518-10) and tubing size no. 16. The immobilized enzyme was placed into bioreactor column (Ø = 2.5 cm, h = 10 cm, v = 49 cm^3) and the dye simulating textile effluent (100 mgL^{-1}) was flowed from Erlenmeyer flask (100 mL) into the beads with flow rate 1.5 mL/min.

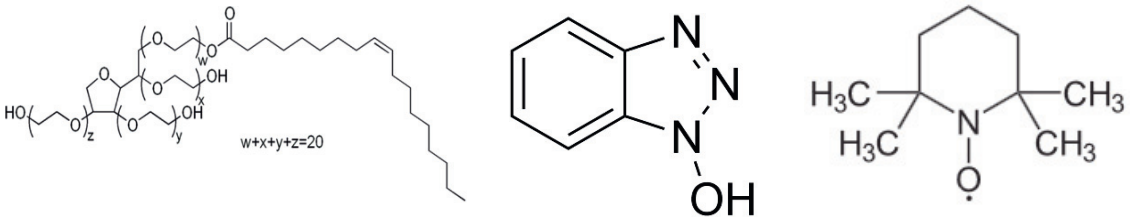

Fig. 1 Chemical structure of enzyme mediators used in this study. Tween 80 (i), HBT (ii), and TEMPO (iii)

Dye decolorization was determined by monitoring the decrease in the absorbance at the wavelength of the maximum absorbance of each dye: LB16 (λmax = 610 nm), RRV9 (λmax = 555.5 nm), RRN4 (λmax = 606 nm). The absorbance of the dye solution was monitored for 1, 2, 3, 6, 12, 24, and 48 h reaction. The decolorization efficiency (R, %) was calculated as the following equation (1).

$$R = (1 - (A \text{ observed})/(A\ initial))x\ 100\% \quad (1)$$

where, A_{initial} is the initial absorbance, and A_{observed} is observed absorbance. Experimental design of vertical bioreactor is shown in Fig. 2.

2.5. Sequential dye decolorization

Sequential batch decolorization was carried out to evaluate the longevity of the beads. For this purpose, the effects of glutaraldehyde addition on five sequential cycles of decolorization were evaluated. The first cycle was run using bioreactor system with dye concentration of 100 mg/L. At the end of each cycle (interval of 360 min), the dye simulating textile effluent was substituted and the beads was washed with 50 mM malonate buffer (pH 4.5) before starting for the next cycle.

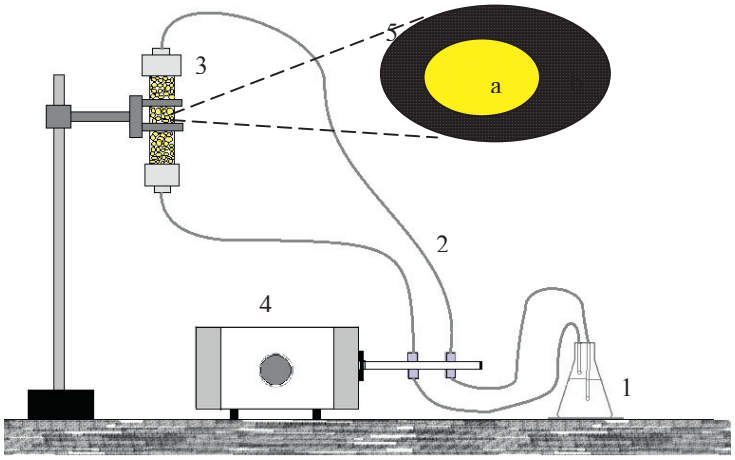

Fig. 2 Experimental design of vertical bioreactor system for decolorization of textile dyes by immobilized enzymes.

3. Results and Discussion

3.1. Decolorization by immobilized enzyme

Dye decolorization of LB16, RRV9, and RRN4 by immobilized enzyme from *T. versicolor* U97 or *Pestalotiopsis* sp. NG007 was first evaluated. Data of the decolorization for 24 h was shown in Table 1. Strong decolorization of LB16 was obtained when immobilized enzyme from U97 was employed. However, no significantly different of decolorization efficiency was observed when the two immobilized enzymes were employed to RRV9 or RRN4. In general, capacity of enzyme to decolorize one dye may be different for other dyes. Therefore, chemical structure of dyes as well as type of dyes influenced the removal efficiency by the enzymes. In this study, decolorization efficiency was decreased in the order LB16 > RRV9 and > RRN4.

Initial pH condition significantly affected the decolorization by the immobilized enzyme, specifically by U97. Decolorization efficiency generally decreased at pH 8.2, except in LB16 by NG007. The decreasing of the decolorization might be due to the decreasing of the enzymatic activities of beads. Under pH 4.5 the ligninolytic activities may involve more optimum to decolorize dyes than at pH 8.2 which is basic condition. It was reported that most basidiomycetes and their enzyme worked significantly in decolorization of textile dyes at ligninolytic condition [6, 7]. In addition, the pH of the environment may affect the mass transfer into the bead caused by the influence of the transport of bulk H^+ion; therefore, it affects the decolorization rate [8].

Efficiency decolorization of textile dyes can also be attributed to the enzyme activities involved in the reaction. This study showed that the efficiency of immobilized enzyme from NG007 to decolorize textile dyes was lower than that of U97 due to this ligninolytic activity characteristic. *T. versicolor* U97 is a basidiomycete fungus mainly produced manganese peroxidase (MnP), laccase, and lignin peroxidase (LiP) that important for removing pollutant such as Remazol Brilliant Blue R (RBBR) and 1,1,1-trichloro-2,2-bis(4-chlorophenyl)-ethane (DDT) in the environment [9]. However, NG007 is an ascomycete fungus with small activities of MnP, laccase, and LiP [10,11]. Although the ligninolytic activities of NG007 were low, the remarkable decolorization was observed by the immobilized enzymes. Some ascomycetes fungi decolorized dyes by an alternative mechanisms such as participation of P450-monooxygenases [12].

Table 1. Decolorization of textile dyes by immobilized enzymes over 24 h.

Immobilized enzyme	Dyes	Decolorization (%) at pH 4.5	at pH 8.2
Pestalotiopsis sp. NG007	LB16	53	56
	RRV9	48	44
	RRN4	44	45
T. versicolor U97	LB16	84	69
	RRV9	51	47
	RRN4	46	45

3.2. Flow rate dependent

Interaction of dyes with the bead is the important factor considered to the success of decolorization. In this study, the flow rate of reaction influenced the decolorization efficiency by immobilized enzyme (Fig. 3). Generally, the faster the flow rate, the lower the biodecolorization rate. For all dyes investigated in this study, when 10 ml/min of flow rate was employed, the biodecolorization was the lowest. In contrast, when the flow rate was 1.0 or 1.5 ml/min, the biodecolorization was optimum. In LB16, the optimum was obtained at the flow rate 1.5 mL/min. However, in RRV9 or RRN4 no significant result was obtained when the flow rate 1.0 or 1.5 mL/min was employed. Therefore, flow rate 1.5 mL/min was used for further investigation.

Biodecolorization of textile dyes using bioreactor had a higher efficiency than in a liquid medium. In liquid medium, the immobilized enzyme decolorized the dyes maximum 36% of the dyes. However, when it was applied in bioreactor, the biodecolorization enhanced to 49 – 100% by flow rate 1.5 mL/min. We suggest that decreasing efficiency in liquid medium is caused by the over-load capacity of the beads when directly applied to dyes.

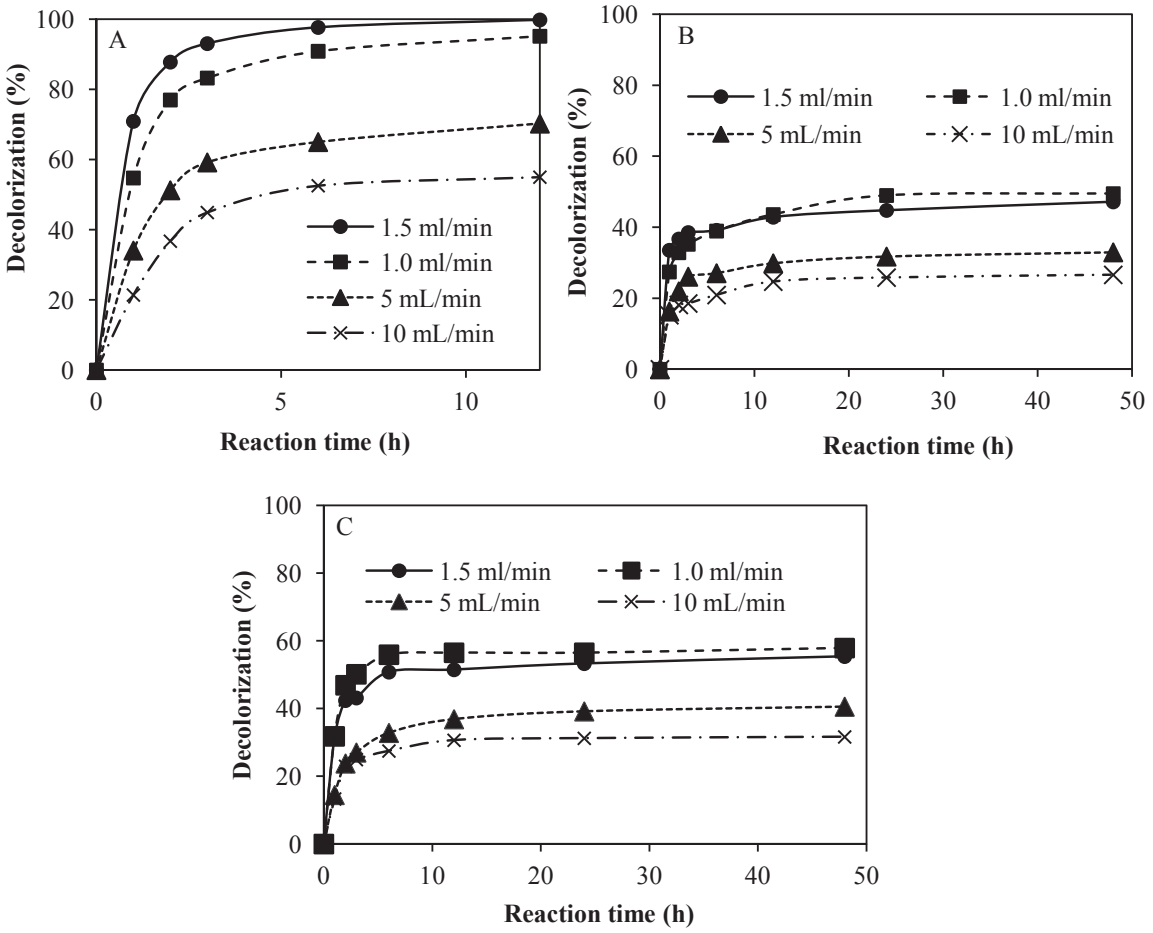

Fig. 3 Effect of flow rate on biodecolorization efficiency of LB16 (A), RRV9 (B), and RRN4 (C) by immobilized enzyme of *Trametes versicolor* U97.

However, these constraints can be overcome by periodically dropped of the dye effluent to the bead with small volume in bioreactor. When the flow rate was fit with the time of reaction, the decolorization reached the optimum condition. In this study, the optimum flow rate was 1.5 mL/min. When the drop of dyes fell on the beads, the reaction was started and enough to be decolorized before another drop came. However, the optimum flow rate may be different for various dyes and immobilized enzymes, and critically influenced by a combination of mass transfer and the capacity of immobilized enzyme to degrade dyes.

3.3. Effect of enzyme mediators

Addition of ligninolytic enzyme mediators such as Tween 80 (1%), HBT (1 mM) or TEMPO (1 mM) generally enhanced the decolorization efficiency for the three textile dyes by two immobilized enzymes either at pH 4.5 or 8.2 (Fig. 4 - 6). Addition of Tween 80 in the presence of Mn^{2+} promoted the oxidation by the enzyme. Tween 80 provides an unsaturated fatty acid chain that may be readily oxidized to form aperoxide which was subsequently turned into a peroxyl radical. This radical was the actual oxidant for the oxidation of the dyes [13]. In many research, addition of HBT has enhanced the decolorization of textile dyes by fungal and enzymes treatment [14, 15, 16, 17].

On the other hand, TEMPO is one of laccase mediators with `stable` *N*-oxyl radical that involves the most effective oxidation of some non-phenolic substrates [18].

Immobilized enzyme from *T. versicolor* U97 had a greater effect of enhancement when 1 mM TEMPO was put in second layer of the beads. Conversely, the most efficient decolorization of textile dyes by immobilized enzyme from *Pestalotiopsis* sp. NG007 was obtained when 1% Tween 80 was employed. Different behavior of immobilized enzyme can be due to the enzyme activities involved in the beads.

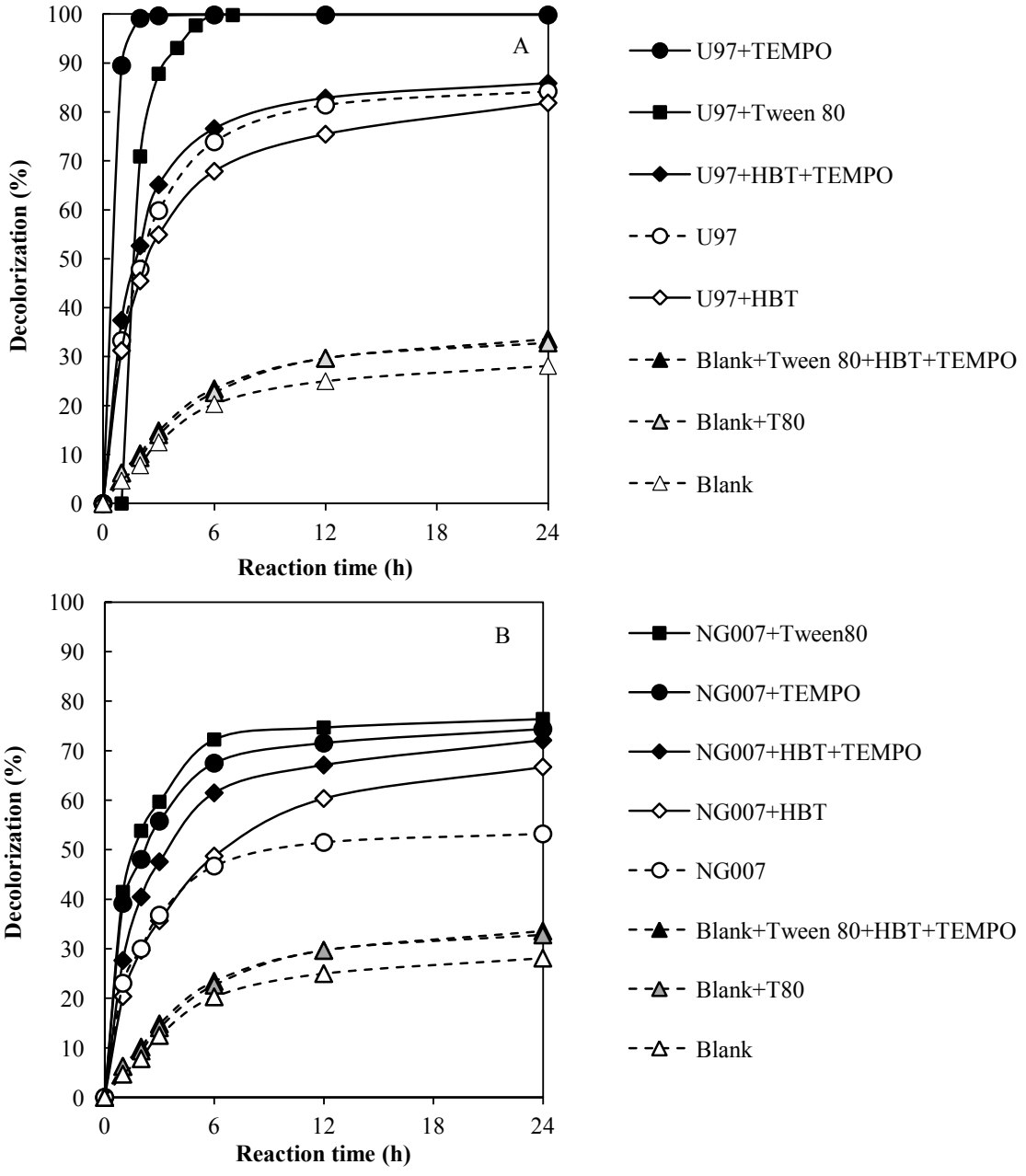

Fig. 4 Effect of various mediators on biodecolorization of LB16 by immobilized enzymes from *Trametes versicolor* U97 (A) and *Pestalotiopsis* sp. NG007 (B) in bioreactor system.

In LB16, the decolorization significantly improved when mediators were used (Fig. 4). In immobilized enzyme of U97, the accelerated decolorization was obtained by the addition of TEMPO (100% decolorization for 3 h reaction) followed by Tween 80 (100% for 6 hours). Unfortunately, addition of HBT could not improve the decolorization rate since it was decreased by 4% compared to the control (without addition of HBT). In addition, when the mixture of HBT and TEMPO was employed to the beads, no significant improvement was observed due to the negative effect of HBT. Approximately 28-32% of dyes were decolorized due to the absorption processes by the blank alginate, by means the decolorization of dyes was due to the enzymatic degradation process.

In contrast with U97, immobilized enzyme of NG007 accelerated the decolorization of LB16 by addition of Tween 80 (72% for 6 hours) followed by TEMPO (67% for 6 hours). The addition of HBT did not show a negative effect to decolorization but the efficiency was lower than the addition of Tween 80 or TEMPO. In addition, when the mixture of HBT and TEMPO was added to the beads, the decolorization was still lower than the addition of TEMPO alone. It can be suggested that the biodecolorization of dyes is due to the enzymatic degradation process because of the low absorption by the blank alginate.

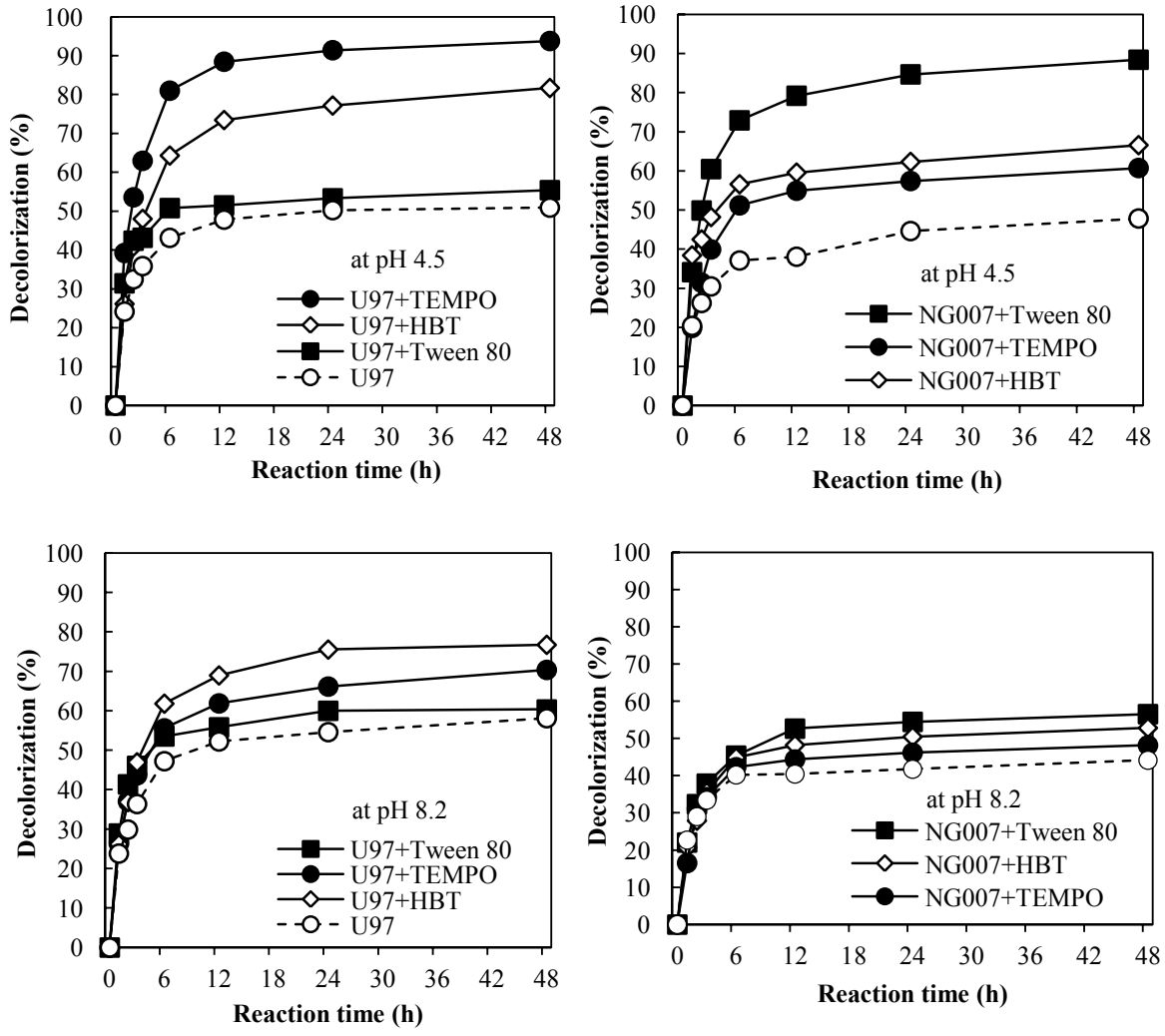

Fig. 5 Effect of various mediators on biodecolorization of RRV9 by immobilized enzymes of *Trametes versicolor* U97 and *Pestalotiopsis* sp. NG007 in bioreactor system at pH 4.5 or at pH 8.2.

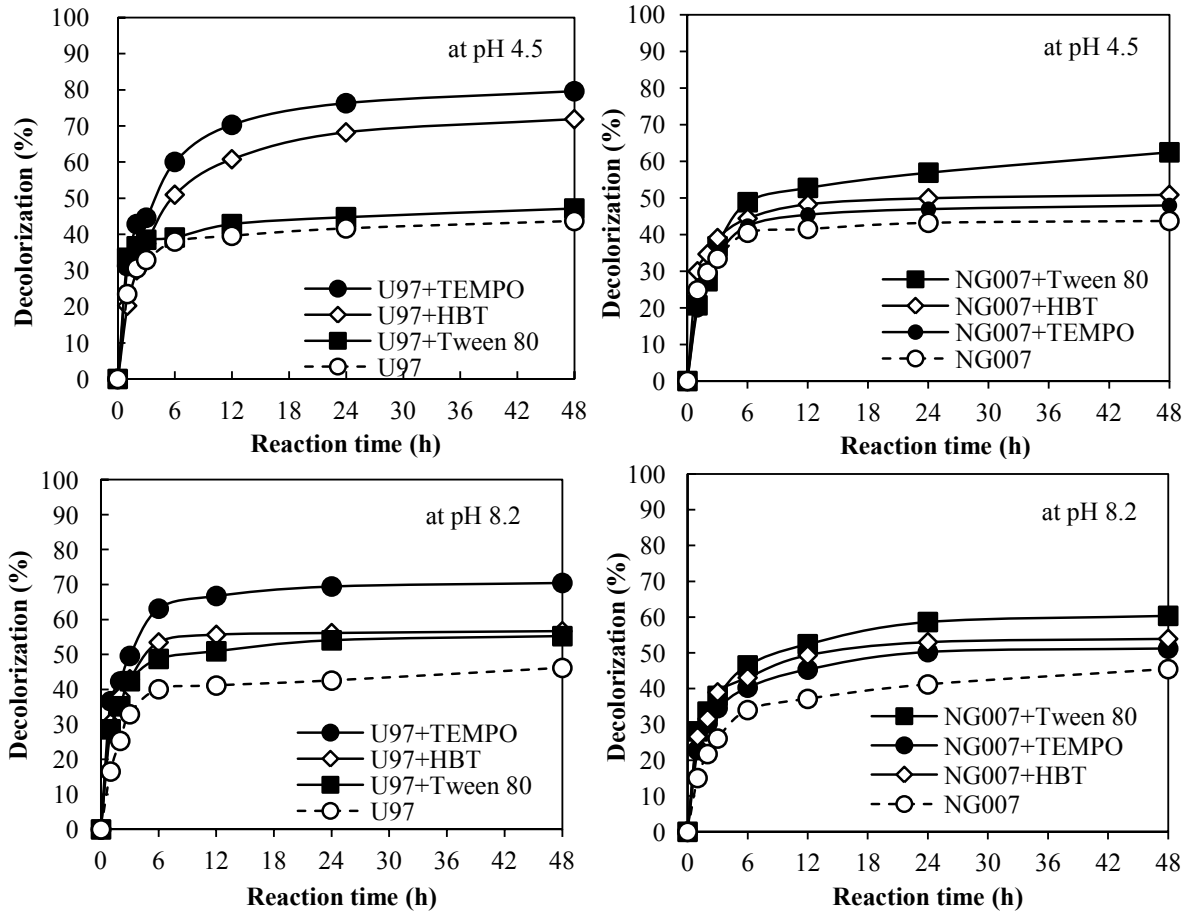

Fig. 6 Effect of various mediators on biodecolorization of RRN4 by immobilized enzymes of *Trametes versicolor* U97 and *Pestalotiopsis* sp. NG007 in bioreactor system at pH 4.5 or at pH 8.2.

The same effect of mediator was also observed in RRV9 and RRN4. When the beads was applied to dyes at pH 4.5, addition of TEMPO in U97 beads, enhanced the decolorization by 84% and 82% for RRV9 and RRN4, respectively. The enhancement, however, decreased when the beads was applied to the pH of dyes 8.2 (21% for RRV9 and 52% for RRN4, respectively). In NG007, addition of Tween 80, enhanced the decolorization by 85% of RRV9 and 43% of RRN4, respectively, at pH 4.5. The enhancement was decreased when applied to the pH 8.2 to become 28% of RRV9 and 32% of RRN4, respectively. These results confirmed that the characteristic and chemical structure of dyes affected the effectiveness of mediator for enhancing decolorization by the beads. The effectiveness of mediators on decolorizing the dyes was decreased in the order LB16 > RRV9 > RRN4, respectively.

3.4. Sequential dye decolorization

Immobilization allows both their reuse for continuous process and the stability of enzyme upon wide pH, temperature range and several of dyes [19]. Therefore, the longevity of the beads is important for the efficient strategy of textile dyes decolorization. In this research, the longevity of the beads was performed by reaction with 0.6% glutaraldehyde for 4 h in ice bath reactor. The result showed that glutaraldehyde increased the longevity of the beads although the efficiency decolorization was decreased in the first cycle when compared with the control (without reaction with glutaraldehyde) (Fig. 7).

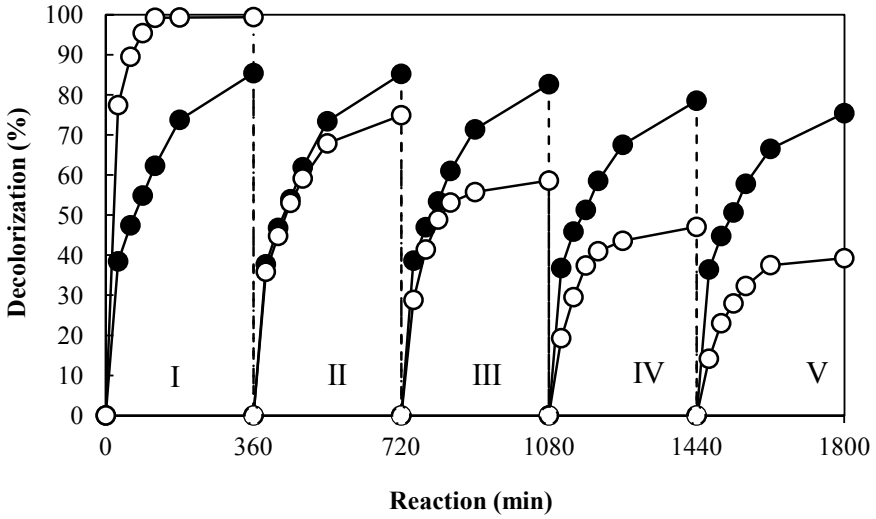

Fig. 7 Sequencing batch decolorization of 100 mgL-1 LB16 by immobilized enzyme of *T. versicolor* U97 in the presence of 1 mM TEMPO using bioreactor system and treated by glutaraldehyde (filled cycle) or without addition of glutaraldehyde (opened cycle).

When sequential batch analysis was performed in bioreactor system, the efficiency decolorization of the beads was drastically decreased in the second cycle and attained only near 40% decolorization in the last cycle. However, reaction of the beads with glutaraldehyde maintained the decolorization in the last cycle at 75%. The results suggest that double layer immobilized enzyme decolorization using bioreactor system is a terrific potential strategy for treating the textile dyes effluents.

4. Conclusion

The immobilized enzymes from fungi showed high decolorization efficiency when they were applied in a vertical bioreactor system. The addition of specific mediators for enzymatic process enhanced the decolorization of dyes by particular immobilized enzymes. The longevity of the beads can be improved by the reaction with glutaraldehyde 0.6% which is important for the reusability of the beads. This study suggests that application of immobilized enzyme in a vertical bioreactor system is a potential tool to improve the biodecolorization of textile dyes from industrial effluents.

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